

## T helper cell cytokine profiles after endurance exercise

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**T HELPER CELL CYTOKINE PROFILES FOLLOWING ENDURANCE EXERCISE**

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**ABSTRACT**

Endurance exercise can cause immunosuppression and increase the risk of upper respiratory illness. The present study examined changes in the secretion of T helper (Th) cell cytokines following endurance exercise. Ten highly-trained road cyclists (mean  $\pm$  SEM: age  $24.2 \pm 1.7$  yr; height  $1.82 \pm 0.02$  m; body mass  $73.8 \pm 2.0$  kg; peak oxygen uptake  $65.9 \pm 2.3$  ml.kg<sup>-1</sup>.min<sup>-1</sup>) performed 2 h of cycling exercise at 90 % of the second ventilatory threshold. Peripheral blood mononuclear cells were isolated, and stimulated with phytohemagglutinin. Plasma cortisol concentrations and the concentration of Th1/Th2/Th17 cell cytokines were examined. Data were analysed using both traditional statistics and magnitude-based inferences. Results revealed a significant decrease in plasma cortisol at 4 h to 24 h post-exercise, compared to pre-exercise values. Qualitative analysis revealed post-exercise changes in concentrations of plasma cortisol, IL-2, TNF, IL-4, IL-6, IL-10, and IL-17A compared to pre-exercise values. A Th1/Th2 shift was evident immediately post-exercise. Furthermore, for multiple cytokines including; IL-2 and TNF (Th1), IL-6 and IL-10 (Th2), and IL-17 (Th17) no meaningful change in concentration occurred until more than 4 hours post-exercise, highlighting the duration of exercise-induced changes in immune function. These results demonstrate the importance of considering ‘clinically’ significant versus statistically significant changes in immune cell function following exercise.

## 1 INTRODUCTION

2 Prolonged endurance exercise is associated with increased incidence of upper respiratory  
3 illnesses (URIs) in elite athletes (Heath and others 1991; Peters and others 1996; Mackinnon  
4 and others 1996; Nieman and others 1990). This increased rate of URIs may be observed  
5 following acute bouts of prolonged heavy- to severe-intensity exercise (e.g., marathon  
6 running, triathlon, road cycling) and periods of increased training load, potentially inducing a  
7 chronic stress response (Walsh and others 2011a). The aetiology of exercise-induced URIs  
8 appears to be multifactorial, as the associated stress response involves many different cell  
9 types, pathways and proteins of the immune system (Walsh and others 2011b).

10  
11 Stress-related changes in immune system function are typically mediated through activation  
12 of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis.  
13 Activation of the SNS induces catecholamine release, with a delayed release of  
14 glucocorticoids. These stress hormones alter immune function after acute exercise and during  
15 periods of heavy training (Pedersen and others 1998), in response to acute and chronic  
16 psychological stress (Cohen and others 1991), and following surgery (Rosenberger and others  
17 2009). The effects of exercise-induced stress hormones on T helper cell function during and  
18 after prolonged heavy-intensity exercise has not been fully elucidated.

19  
20 Glucocorticoids and catecholamines influence T helper cell (Th) cytokine release, principally  
21 by stimulating glucocorticoid and  $\beta 2$  adrenergic receptors, respectively (Calcagni and  
22 Elenkov 2006). In turn, these stress hormones influence the activity of cellular (Th1),  
23 humoral (Th2), and mucosal (Th17) branches of the immune response (Calcagni and Elenkov  
24 2006; Elenkov 2004). Specifically, cortisol, adrenaline and noradrenaline may suppress  
25 cytokine release from antigen presenting cells and Th1 cells, while up-regulating cytokine  
26 production from Th2 cells, thereby causing a shift toward Th2 cytokine production  
27 (Steensberg and others 2001; Lancaster and others 2004). The balance between Th1 and Th2  
28 immunological responses is very important in maintaining optimum immune health. The Th1  
29 pathway primarily acts against intracellular pathogens, particularly viruses and bacteria,  
30 while the Th2 pathway is believed to protect against extracellular pathogens such as parasites  
31 (Kidd, 2003). Previous research has extensively examined changes in the balance of Th1 and  
32 Th2 cytokines in plasma in the first 1–2 h after exercise (Suzuki and others 2002). By  
33 contrast, there is less research published concerning changes in T helper cytokine secretion

(including Th17 cytokines) during recovery from prolonged heavy exercise. While these changes are transient, it may have implications related to potential immune suppression following prolonged heavy intensity exercise (Nieman, 2000).

Exercise-induced glucocorticoid release occurs during prolonged exercise (Pedersen and others 1998). An increase in glucocorticoid concentration after exercise may influence T helper cytokine secretion differently to exercise of moderate duration and intensity, due to the large effect of changes in cortisol on immune function (Walsh and others 2011b; Pedersen and others 1998). Th17 cells play a key role in mucosal host defence of the upper respiratory and gastrointestinal tracts against a myriad of airborne antigens (Korn and others 2009; Liang and others 2006; Yao and others 1995). Functioning as part of the “first line of defence” in the mucosa, Th17 cytokines have been linked to increased polymeric immunoglobulin receptor (IgR) expression, saliva SIgA and human  $\beta$ -defensin 2 release (Johansen and others 1999; Jaffar and others 2009; Kao and others 2004; Brandtzaeg and others 1999). These results demonstrate that Th17 cytokine pathways are influential in the innate immune response in the mucosa. However, there is little evidence available about changes in Th17 cytokines after exercise.

The aim of the present study was to examine the effect of prolonged heavy exercise on plasma cortisol concentration, and Th1, Th2, and Th17 cytokine release. We hypothesised that the exercise-induced stress would increase plasma cortisol concentration after exercise, resulting in the release of IL-17 and a shift from secretion of Th1 to Th2 cytokines.

## **MATERIALS AND METHODS**

### **Subjects**

Ten well-trained male cyclists volunteered to participate in the present study (mean  $\pm$  SEM: age  $24.2 \pm 1.7$  yr; height  $1.82 \pm 0.02$  m; body mass  $73.8 \pm 2.0$  kg; peak oxygen uptake  $65.9 \pm 2.3$  ml.kg<sup>-1</sup>.min<sup>-1</sup>). Subjects were excluded if they exhibited any cardiovascular, metabolic, neurological, immunological or autoimmune disorders. The cyclists provided written informed consent for participation in the study, which was approved by the Bond University Human Research Ethics Committee.

### **Experimental Design**

The cyclists visited the laboratory on three occasions; for a screening/familiarisation session, a maximal exercise test, and a constant load test with blood sampling before and after exercise. Peak oxygen consumption ( $\dot{V}O_{2peak}$ ) was determined during a graded exercise test in which the cyclists were allowed to choose a preferred cadence within the range of 70–90 rev.min<sup>-1</sup>. The test was performed on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). The cyclists commenced cycling at 80 W for 5 min; thereafter intensity increased by 30 W/min until volitional fatigue and/or when cadence could not be maintained at a minimum 70 rev.min<sup>-1</sup>. The criteria to determine maximal efforts at the end of a test were: a plateau in  $\dot{V}O_2$  (defined as an increase in  $\dot{V}O_2$  values of less than 1.5 ml.kg<sup>-1</sup>.min<sup>-1</sup> between two consecutive 1-min periods in the final part of the test), a maximal value of respiratory exchange ratio  $\geq 1.1$ , a maximal heart rate value above 95 % of the age-predicted maximum (220-age) (Doherty and others 2003). Each cyclist's second ventilatory threshold (VT<sub>2</sub>) was determined from the gas-exchange data collected during incremental exercise (ParvoMedics' TrueOne® 2400, Utah, USA), using the ventilatory equivalents methods (Wasserman and McIlroy 1964).

The cyclists refrained from exercise for 48 h prior to the maximal exercise test and experimental trial, and avoided consumption of alcohol or caffeine in the previous 24 h, and during the exercise and post-exercise sample period. Pre-exercise blood samples were collected from the cyclists after an overnight fast. The cyclists then consumed a standardised breakfast (2000 kJ) of cereal, milk, and juice and commenced the exercise trial at 0800 hrs. The cyclists performed exercise for 2 h at 90 % VT<sub>2</sub> (Monark Ergonomic 828 E, Sweden). Cardiac rate and rhythm were monitored continuously throughout exercise, and cardiac rate was recorded each minute. Pulmonary gas exchange was sampled for 4 min at 20-min intervals to ensure that the cyclists were exercising at the work rate associated with 90 % VT<sub>2</sub> and that no drift in oxygen uptake occurred. The cyclists were allowed to drink water *ad libitum* during exercise. The cyclists were provided with a standardised meal (4500 kJ) of sandwiches, and a milkshake at 2.5 h post-exercise.

### **Blood collection**

Venous blood samples were collected pre-exercise, immediately post-exercise, 2 h, 4 h, 6 h, 8 h, and 24 h post-exercise. Blood was collected into tubes containing K<sub>3</sub>EDTA (Becton Dickinson, New Jersey, USA). One tube was immediately centrifuged at 1000 × g for 15 min.

Plasma was stored in aliquots at -80°C. The remaining blood sample was used for the cytokine stimulation assay.

#### **Plasma cortisol analysis**

Plasma cortisol was analysed by ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC MS/MS) described elsewhere (McWhinney and others 2010). Briefly, plasma samples were treated with 1 M HCl at room temperature to displace cortisol and cortisone from binding proteins. To each sample, the multi-analyte internal standard (d4-dexamethasone, d2-11-deoxycortisol and d2-cortisone) and H<sub>2</sub>O were added before loading onto activated Oasis HLB 1 cc (30 mg) solid phase extraction cartridges (Waters, Milford, USA). Samples were eluted with 100 % ethyl acetate, dried at 50°C and reconstituted in 45 % methanol with 2 mmol/L ammonium acetate and 0.1 % formic acid. The flow rate of the UHPLC analysis was 0.4 ml/min, sample injection volume 20 µl, and column temperature 50°C. The tandem mass spectrometer was operated in positive mode electron spray ionisation. The total analytical run time on the UHPLC MS/MS was 3 min.

#### **Flow cytometry**

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Milan, Italy) and then counted (Countess<sup>®</sup> Automated Cell Counter, Life Technologies, Carlsbad, CA). Cells were cultured at a concentration of  $1 \times 10^6$  cells/ml and stimulated with 1 µg/ml of the mitogen phytohemagglutinin (PHA) for 72 h. Following incubation, the supernatant was removed and stored at -80°C for subsequent analysis. Th1, Th2 and Th17 cytokine concentrations were quantified using a commercially available cytometric bead array kit (BD Pharmingen, San Diego, CA) to measure the concentrations of IL-2, IL-4, IL-6, IL-10, TNF, INF-γ and IL-17A from supernatant of stimulated samples. In the absence of stimulation with PHA, the concentrations of these cytokines were not detected.

#### **Statistics**

Prior to statistical analysis, all data were tested to determine if they were normally distributed. Data that were normally distributed (Figure 1) were analysed using one-way ANOVA with repeated measures to determine any change over time, with a Bonferroni correction applied post hoc when significance was evident. These data are presented as mean ± SEM. Data that were not normally distributed (Table 3) were analysed using the non-parametric Friedman's

ANOVA and Wilcoxon signed-rank test. These data are presented as median  $\pm$  interquartile range. The Bonferroni correction was used for all multiple comparisons, with significance set at  $p < 0.008$  ( $p < 0.05$  divided by the number of time points). All statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, USA). Data was further analysed for ‘clinical’ significance using magnitude-based inferences (Hopkins and others 2009). All qualitative analyses based on effect size were conducted using a modified statistical spreadsheet (available at [www.sportsci.org/resource/stats/xPostOnlyCrossover.xls](http://www.sportsci.org/resource/stats/xPostOnlyCrossover.xls)) (Hopkins 2006). Measures were log-transformed before analysis to symmetrically distribute the non-normally distributed data. Magnitudes of effects were determined by standardisation of the log-transformed variable. Between-time point standardized differences and effect sizes for all values were calculated using the pooled standard deviation (Cohen 1998). Threshold values for Cohen Effect Size statistics were  $> 0.2$  (small),  $> 0.5$  (moderate), and  $> 0.8$  (large). For between-time point comparisons, probabilities were calculated to establish whether the true (unknown) differences were lower, similar or higher than the smallest worthwhile difference or change. The smallest worthwhile difference for each variable was derived by standardisation: 0.20 multiplied by the pooled between-subject standard deviations at each time point compared to pre-exercise, based on Cohen’s Effect Size principle (Cohen 1998). Quantitative chances of higher or lower differences were evaluated qualitatively as follows:  $< 1\%$ , almost certainly not;  $1\text{--}5\%$ , very unlikely;  $5\text{--}25\%$ , unlikely;  $25\text{--}75\%$ , possible;  $75\text{--}95\%$ , likely;  $95\text{--}99\%$ , very likely;  $> 99\%$ , almost certain. If the chance of higher or lower differences was  $> 5\%$ , the true difference was assessed as unclear. Otherwise, the change was interpreted as the observed chance (Hopkins and others 2009).

## RESULTS

### Physical characteristics and exercise values

Physical characteristics of the cyclists are presented in Table 1. Mean heart rate for the 2 h exercise bout was  $161 \pm 3$  beats.min<sup>-1</sup>, and mean exercise intensity associated with 90 % VT<sub>2</sub> occurred at  $75.1 \pm 0.9\%$  of  $\dot{V}O_{2\text{peak}}$ .

<insert Table 1 here>

### Plasma cortisol



Figure 1 displays plasma cortisol concentration following prolonged heavy-intensity exercise. There was no statistically significant change immediately or 2 h post-exercise. Plasma cortisol concentration significantly decreased 4 h post-exercise ( $p < 0.001$ ), when compared with the resting pre-exercise concentration, and remained significantly decreased at 6 h post-exercise ( $p < 0.01$ ), 8 h post-exercise ( $p < 0.001$ ) and 24 h post-exercise ( $p < 0.01$ ).

<insert Figure 1 here>

Magnitude-based inferences revealed that compared to pre-exercise, plasma cortisol concentration was likely higher immediately post-exercise. The chances that the true difference in cortisol was higher/trivial/lower were 94/5/1%. Cortisol was very likely lower at 2 h post-exercise (1/2/97 %). Furthermore, compared to pre-exercise, cortisol was almost certainly lower at 4 h (0/0/100 %), 6 h (0/0/100 %), and 8 h (0/0/100 %). At 24 h post-exercise, cortisol was likely lower (4/12/84 %) than pre-exercise values (Table 2).

<insert Table 2 here>

### **T helper cell cytokines**

There were no statistically significant changes in PHA-stimulated production of the T helper 1 cytokines IL-2, TNF, and IFN- $\gamma$ , T helper 2 cytokines IL-4 and IL-10, and T helper 17 cytokine IL-17A following exercise (Table 3). A significant time effect was observed for IL-6; however, post hoc analysis revealed no significant change at individual time points after exercise compared with the pre-exercise IL-6 concentration.

<insert Table 3 here>

Table 4 displays changes in Th1 cytokine concentrations. Compared with pre-exercise values, the concentration of IL-2 was likely higher than pre-exercise at 4 h (85/15/0 %) and 8 h (90/10/0 %) post-exercise. The concentration of TNF was also likely higher than pre-exercise values at 4 h post-exercise (82/17/1 %). Changes in IFN- $\gamma$  concentration were unclear at every time point post-exercise.

<insert Table 4 here>

Compared to pre-exercise, the concentration of IL-4 was likely higher immediately post-exercise (79/18/3 %), while the difference in IL-4 during the remaining recovery period were unclear (Table 5). Compared to pre-exercise, the IL-6 concentration was possibly higher at 4 h (68/29/3 %), possibly lower at 6 h (4/42/54 %), and likely higher at 8 h post-exercise (94/6/0 %). Compared to pre-exercise, IL-10 concentration was likely higher at 4 h post-exercise (77/23/0 %).

<insert Table 5 here>

IL-17A concentration compared to pre-exercise was possibly higher than pre-exercise at 8 h post-exercise (73/25/2 %) (Table 6).

<insert Table 6 here>

## DISCUSSION

This is the first study to analyse changes in Th1, Th2 and Th17 cytokine production during recovery from exercise. It is established that exercise intensity and duration influence the cortisol response to exercise. Previously, cycle exercise of comparable intensity (~75 %  $\dot{V}O_2$ peak) and duration (2 h) resulted in a significant increase in cortisol concentration immediately post-exercise (Nieman and others 2007). Although traditional data analysis in the present study did not reveal any statistically significant increase in plasma cortisol immediately post-exercise, quantitative methods used suggested an increase in plasma cortisol concentration was likely, and given results of previous research of similar exercise duration and intensity, this result would be expected.

Plasma cortisol concentration decreased below pre-exercise levels from 4 h to 24 h post-exercise. While the initial decrease (which occurred early in the afternoon) may be explained by diurnal variation (Dhabhar and others 1994), plasma cortisol concentration remained below resting values at 24 h post-exercise. This low plasma cortisol concentration may be clinically important when considering the repetitive nature of endurance training day after day. This result may indicate that the cyclists were well rested following the exercise bout,

and would have been able to respond positively to an additional bout of exercise on the second morning.

Using traditional data analysis, there were no statistically significant changes in T helper cell cytokine secretion at any time point during the post-exercise period in the present study (Table 3). However, analysis using magnitude-based inferences suggested a multitude of changes following exercise. Compared to pre-exercise, IL-2 (Th1) production was likely higher at 4 h and again at 8 h post-exercise (Table 4), while TNF (Th1) production was likely higher at 4 h post-exercise (Table 4). IL-4 (Th2) production was likely higher immediately post-exercise (Table 5), while IL-6 (Th2) production was possibly higher at 4 h, possibly lower at 6 h, and likely higher at 8 h post-exercise (Table 5). IL-10 (Th2) production was likely higher at 4 h post-exercise (Table 5), while IL-17A (Th17) production was possibly higher at 8 h post-exercise (Table 6).

Previously, intracellular cytokines IL-2 (Th1), IFN- $\gamma$  (Th1), and IL-4 (Th2) produced by CD4<sup>+</sup> T cells were used to define T helper cell types and their functional changes. Using this method, a Th1-to-Th2 shift was described following exercise and hormone infusion (Elenkov 2004; Elenkov 2008; Lancaster and others 2004; Steensberg and others 2001). This ‘shift’ occurs when IL-2- and/or IFN- $\gamma$ -producing T helper cells (Th1) decrease in cell concentration, while IL-4-producing T helper cells (Th2) remain unchanged. In the present study, no change in IL-2 and IFN- $\gamma$  occurred immediately post-exercise (Table 4), while IL-4 concentration increased at the same time point (Table 5). Hence, a shift towards Th2 was observed immediately post-exercise.

Although single intracellular cytokine analysis of T helper cells provides a concise explanation of the Th response to exercise, it neglects to consider the magnitude of cytokines involved in the T helper cell network. Cytokines are not solely secreted from T helper cells, but include cytotoxic T cells, natural killer cells, dendritic cells, macrophages and even skeletal muscle ( Pedersen and Febbraio 2008; Zaldivar and others 2006). In the present study, cytokine secretion both increased and decreased occurred immediately following exercise (IL-4), and up to 8 hours post-exercise (IL-2, IL-6 and IL-17A). This presents an intricate pattern of changes to consider. In particular, at 4 h post-exercise, the Th1 cytokines IL-2 and TNF, and the Th2 cytokines IL-6 and IL-10 were all increased. This concomitant increase

1 and balancing of Th1 and Th2 cytokines has been observed previously by measuring  
2 intracellular cytokine production from unstimulated peripheral blood mononuclear cells  
3 following 30 min of moderate-intensity cycling (Zaldivar and others 2006). In contrast, the  
4 present study examined the functional ability of peripheral blood mononuclear cells to  
5 respond to a stimulus (PHA). Interestingly, the release of multiple cytokines was elevated at 4  
6 h post-exercise. These responses demonstrate that exercise-induced stress can prime  
7 peripheral blood mononuclear cells to respond to stimulation (Dhabhar 2009).

8  
9 Recently, analysis by magnitude-based inference has received greater use in exercise science  
10 and clinical settings. As previously stated, using traditional data analysis, there were no  
11 statistically significant changes in T helper cell cytokine secretion at any time point during  
12 the post-exercise period in the present study (Table 3). However, analysis using magnitude-  
13 based inferences suggested various changes following exercise, which may prove clinically  
14 significant. This approach may provide insights into what are clinically important, yet  
15 statistically non-significant results in physiological and immunological processes. This study  
16 assists in highlighting the importance of considering non-traditional methods for data analysis  
17 when dealing with small sample sizes, with high between-subject variability.

18  
19 The changes that occurred in T helper cell cytokine release suggest a multifactorial,  
20 prolonged response to heavy endurance exercise. The fluctuations in cytokine production that  
21 occurred during recovery from exercise could have important implications for resistance to  
22 illness in athletes. While intracellular cytokine analysis following stimulation provides a  
23 concise explanation of the Th1/Th2 shift, the current study suggests a much more complex  
24 response to exercise.

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4  
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7  
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11

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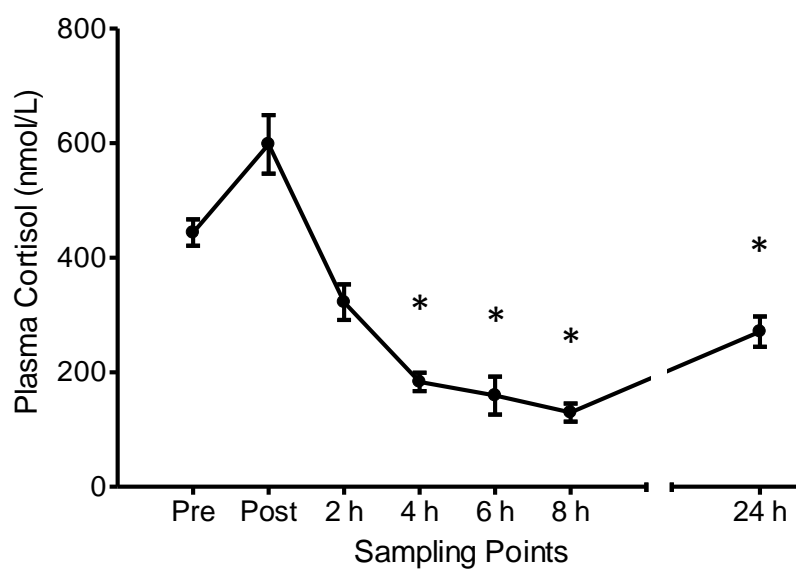


Figure 1. Plasma cortisol concentration before and after prolonged heavy intensity cycling.

Data represent mean  $\pm$  SEM. \*, significantly change compared to 'Pre' ( $p < 0.05$ )

Table 1. Physical characteristics and aerobic capacity of the cyclists

Subject Characteristics (n=10)	Mean $\pm$ SEM
Age (years)	24.2 $\pm$ 1.7
Height (m)	1.82 $\pm$ 0.02
Body mass (kg)	73.8 $\pm$ 2.0
BMI (kg.m <sup>-2</sup> )	22.2 $\pm$ 0.4
Absolute $\dot{V}O_{2peak}$ (L.min <sup>-1</sup> )	4.83 $\pm$ 0.1
Relative $\dot{V}O_{2peak}$ (ml.kg <sup>-1</sup> .min <sup>-1</sup> )	65.9 $\pm$ 2.3
Training (km/week)	292.5 $\pm$ 28.4

Data are mean  $\pm$  standard error of the mean. BMI: body mass index.  $\dot{V}O_{2peak}$ : peak oxygen uptake. Training: volume in kilometres performed during training per week.

1 Table 2. Qualitative analysis of changes in cortisol concentration following prolonged heavy  
 2 intensity exercise

	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
% Difference	39.4	-24.6	-58.3	-63.0	-70.0	-38.8
(90% CL)	(13.2; 65.6)	(-39.8; -9.5)	(-64.7; -52.0)	(-76.7; -49.3)	(-76.8, -63.3)	(-48.0; -29.6)
Effect Size	1.3 (large)	-1.4 (large)	-4.2 (large)	-3.2 (large)	-5.1 (large)	-2.2 (large)
% Chance for value to be higher/trivial/lower than Pre	94/5/1	1/2/97	0/0/100	0/0/100	0/0/100	4/12/84
Qualitative inference	Likely higher	Very likely lower	Almost certainly lower	Almost certainly lower	Almost certainly lower	Likely lower

3 Data presented as percentage difference compared to the pre-exercise concentration. CL:  
 4 Confidence level.

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1 Table 3. T helper cytokine release before and after a bout of prolonged heavy intensity-  
 2 exercise exercise

	Pre-exercise	Post-exercise	2 h	4 h	6 h	8 h	24 h	P value
IL-2	18.9 (3.9, 59.8)	11.5 (2.8, 24.0)	11.2 (2.5, 51.7)	65.8 (8.0, 110.0)	50.7 (10.6, 107.0)	47.4 (13.6, 96.3)	7.1 (1.7, 162.0)	0.141
TNF	2.0 (1.5, 2.4)	1.8 (1.4, 2.0)	1.8 (1.5, 2.1)	2.3 (2.0, 3.3)	2.9 (1.7, 3.9)	2.0 (1.6, 2.6)	1.9 (1.4, 2.8)	0.488
IFN- $\gamma$	1.1 (1.1, 1.3)	1.1 (1.0, 1.2)	1.2 (1.1, 1.4)	1.3 (1.1, 2.4)	1.6 (1.2, 2.3)	1.8 (1.1, 4.8)	1.1 (1.0, 1.4)	0.103
IL-4	2.0 (1.9, 2.1)	2.1 (2.0, 2.2)	2.1 (1.9, 2.3)	2.0 (1.9, 2.2)	2.0 (1.9, 2.1)	2.0 (2.0, 2.2)	1.9 (1.9, 2.0)	0.227
IL-6	347.7 (14.3, 835.8)	142.7 (32.4, 1061.8)	98.3 (30.5, 433.4)	249.9 (112.6, 706.2)	458.4 (125.1, 1006)	718.2 (95.7, 990.3)	131.7 (49.9, 606.0)	0.039
IL-10	4.4 (1.8, 7.5)	2.7 (1.8, 7.2)	4.6 (1.8, 7.4)	5.2 (2.2, 16.1)	11.5 (2.7, 17.0)	6.6 (2.2, 19.3)	3.7 (1.5, 30.0)	0.126
IL-17A	2.8 (2.8, 3.0)	2.9 (2.5, 3.3)	2.7 (2.7, 3.2)	2.8 (2.8, 3.2)	2.8 (2.6, 3.1)	3.0 (2.7, 3.1)	2.8 (2.6, 3.0)	0.500

3 Th1 cytokines: IL-2, TNF, IFN- $\gamma$ . Th2 cytokines: IL-4, IL-6, IL-10. Th17 cytokine: IL-17A.  
 4 Data represent median (1<sup>st</sup> and 3<sup>rd</sup> quartile).

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1 Table 4. Qualitative analysis of changes in Th1 cytokine concentrations following prolonged  
 2 heavy-intensity exercise

	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
<b>IL-2</b>						
% Difference	69.3	-4.0	162.2	142.4	115.3	132.6
(90% CL)	(-75.7; 214.4)	(-33.0; 25.1)	(42.5; 281.9)	(64.0; 220.7)	(34.0; 196.5)	(-41.5; 306.6)
Effect Size (rating)	-0.1 (trivial)	0.04 (trivial)	0.5 (moderate)	0.5 (moderate)	0.3 (small)	0.5 (moderate)
% Chance for value to be higher/trivial/lower than Pre	7/49/44	1/77/22	85/15/0	26/59/15	90/10/1	4/94/2
Qualitative inference	Unclear	Trivial	Likely higher	Unclear	Likely higher	Trivial
<b>TNF</b>						
% Difference	-3.2	80.7	45.4	145.2	11.4	20.5
(90% CL)	(-24.7; 18.2)	(-55.9; 217.4)	(-1.1; 92.0)	(-43.8; 334.3)	(-9.4; 32.2)	(-17.4; 58.4)
Effect Size (rating)	-0.4 (small)	0.5 (moderate)	0.6 (moderate)	0.7 (moderate)	0.1 (trivial)	0.2 (small)
% Chance for value to be higher/trivial/lower than Pre	6/47/47	56/31/14	82/17/1	69/21/11	44/46/10	60/26/14
Qualitative inference	Unclear	Unclear	Likely higher	Unclear	Unclear	Unclear
<b>IFN-<math>\gamma</math></b>						
% Difference	5.2	7.3	11.8	25.5	77.0	21.8
(90% CL)	(-32.7; 43.2)	(-28.2; 42.8)	(-11.9; 35.5)	(-4.6; 55.6)	(-0.0; 154.1)	(-35.5; 79.2)
Effect Size (rating)	-0.5 (moderate)	-0.4 (small)	-0.2 (small)	-0.1 (trivial)	0.2 (small)	-0.03 (trivial)
% Chance for value to be higher/trivial/lower than Pre	11/28/61	11/47/42	18/77/6	45/50/5	50/43/7	33/54/14
Qualitative inference	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear

3 Data presented as percentage difference compared to the pre-exercise concentration. CL:  
 4 Confidence level.

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1 Table 5. Qualitative analysis of changes in Th2 cytokine concentrations following prolonged  
 2 heavy-intensity exercise  
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	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
<b>IL-4</b>						
% Difference	5.4	5.1	4.2	-0.1	2.6	-3.4
(90% CL)	(-0.4; 11.2)	(-3.2; 13.4)	(-3.6; 11.9)	(-4.3; 4.0)	(-3.9; 9.2)	(-8.9; 2.0)
Effect Size (rating)	0.5 (moderate)	0.4 (small)	0.3 (small)	-0.1 (trivial)	0.2 (small)	-0.6 (moderate)
% Chance for value to be higher/trivial/lower than Pre	79/18/3	66/23/10	62/28/11	33/26/41	21/43/35	14/23/62
Qualitative inference	Likely higher	Unclear	Unclear	Unclear	Unclear	Unclear
<b>IL-6</b>						
% Difference	250.8	159.2	640.3	397.5	461.1	359.7
(90% CL)	(79.8; 581.3)	(-123.0; 441.4)	(-195.6; 1476.1)	(109.1; 686.0)	(87.8; 834.5)	(-75.3; 794.7)
Effect Size (rating)	0.1 (trivial)	-0.6 (moderate)	-0.2 (small)	0.2 (small)	0.4 (small)	0.04 (trivial)
% Chance for value to be higher/trivial/lower than Pre	39/54/7	11/60/29	68/29/3	4/42/54	94/6/0	35/64/1
Qualitative inference	Unclear	Unclear	Possibly higher	Possibly lower	Likely higher	Trivial
<b>IL-10</b>						
% Difference	10.9	46.7	63.7	116.5	139.7	153.0
(90% CL)	(-27.8; 49.7)	(-28.5; 121.8)	(19.0; 108.4)	(3.9; 229.1)	(49.5; 229.8)	(10.2; 295.8)
Effect Size (rating)	-0.1 (trivial)	0.4 (small)	0.5 (moderate)	0.6 (moderate)	0.8 (large)	0.9 (large)
% Chance for value to be higher/trivial/lower than Pre	7/69/25	26/70/4	77/23/0	46/43/11	46/53/0	25/72/3
Qualitative inference	Unclear	Trivial	Likely higher	Unclear	Trivial	Trivial

4 Data presented as percentage difference compared to the pre-exercise concentration. CL:  
 5 Confidence level.

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1 Table 6. Qualitative analysis of changes in Th17 cytokine IL-17A concentration following  
 2 prolonged heavy-intensity exercise

	Pre – Post	Pre – 2 h	Pre – 4 h	Pre – 6 h	Pre – 8 h	Pre – 24 h
IL-17A						
% Difference	1.9	-1.2	5.2	4.8	8.4	-2.0
(90% CL)	(-3.8; 7.7)	(-10.3; 7.9)	(-6.4; 16.9)	(-6.5; 16.0)	(-5.3; 22.0)	(-11.4; 7.4)
Effect Size (rating)	0.1 (trivial)	-0.1 (trivial)	0.3 (small)	0.2 (small)	0.4 (small)	-0.4 (small)
% Chance for value to be higher/trivial/lower than Pre	29/62/9	15/42/44	51/35/15	17/30/53	73/25/2	31/32/37
Qualitative inference	Unclear	Unclear	Unclear	Unclear	Possibly higher	Unclear

4 Data presented as percentage difference compared to the pre-exercise concentration. CL:  
 5 Confidence level.

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